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TITLE: Dysregulated microRNA Activity in Shwachman-Diamond Syndrome

PRINCIPAL INVESTIGATOR: Carl Novina

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02215-5450

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14. ABSTRACT

Shwachman-Diamond Syndrome (SDS) is an inherited bone marrow failure primarily affecting myeloid development. Because the affected cells are rare and heterogeneous, the altered genetic networks in vivo remain unknown. The central goal of this grant is to define transcriptional signatures of bone marrow failure in SDS using single cell RNA-seq of patient cells. We will analyze these datasets to test the novel hypothesis that reduced microRNA activity contributes to hematopoietic dysfunction in SDS. To date, we have sequenced ~300 hematopoietic stem and progenitor cells (HSPC) from normal donors and SDS patients and established, to our knowledge, the first lineage maps of early hematopoiesis at single cell resolution. We have used single cell statistical frameworks such as MAST, SCDE and WGCNA to identify SDS-specific gene expression patterns that are common to all HSPC or restricted to particular lineages. Ongoing and future work includes 1) annotation of differentially expressed genes to hematopoietic phenotypes in cellular and animal models of SDS, 2) profiling thousands more HSPC using high-throughput Drop-seq, and 3) generation of microRNA expression profiles from HSPCs to be overlaid onto mRNA profiles.

15. SUBJECT TERMS

Single cell RNA-seq; bone marrow failure; hematopoiesis; myelopoiesis; targeted RNA-seq

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Table of Contents

Introduction

Patients with Shwachman-Diamond Syndrome (SDS) suffer from hematopoietic abnormalities including neutropenia, thrombocytopenia, pan-anemia and myelodysplasia (MDS) with progression to acute myeloid leukemia (AML). The affected cell types and altered genetic networks *in vivo* remain unknown, primarily due to the rarity and heterogeneity of bone marrow progenitors. Our overarching goal is to define transcriptional signatures of bone marrow failure in SDS by performing single cell RNA sequencing (RNA-seq) on freshly isolated patient cells. These studies may lead to the development of targeted molecular therapies as an alternative to bone marrow transplant for the treatment hematopoietic dysfunction in SDS.

Keywords

Single cell RNA-seq; bone marrow failure; hematopoiesis; myelopoiesis; targeted RNA-seq

Accomplishments

What were the major goals of the project?

Aim 1. Define the molecular basis for the SDS myelopoiesis defect at single cell resolution.

Sub-Task 1A: Consent 6 SDS patients under protocol 10-02-0057, and 6 normal donors under protocol 09-04-0167; obtain patient bone marrow samples, purify CD34+ cells. (Start date 9/30/2014; duration 24 months; protocol renewed 11/09/2015 and 8/21/2015, respectively, by local IACUC).
Milestone: Obtain 6 patient and 6 donor samples.
Percent completion: 60%

Sub-Task 1B: Load purified cells onto C1 chips; prepare ~96 single cell RNA-seq libraries per sample; run sequencing reactions. (Start date 9/30/2014; duration 24 months)
Milestone: Generate genome-wide transcriptional profiles of CD34+ cells form normal donors and SDS patients at single cell resolution.
Percent completion: 60%

Sub-Task 1C: Process data; perform bioinformatic analyses. (Start date 3/30/2015; duration 30 months)
Milestone: Identify SDS affected progenitor cells; Define SDS gene expression networks in single cells or subpopulations of CD34+ cells; Predict microRNA-targeted mRNAs that explain pathogenesis of SDS.
Percent completion: 70%

Aim 2. Functionally annotate SDS transcriptomes to myelopoiesis defects.

Sub-Task 2A: Obtain 2-4 frozen mononuclear cells from SDS patient repository under protocol 10-02-0057 and 2-4 normal donors under protocol 09-04-0167. (Start date 9/30/2015; duration 24 months; protocol renewed 11/09/2015 and 8/21/2015, respectively, by local IACUC).
Milestone: Obtain 2-4 SDS patient and 2-4 donor samples.
Percent completion: 20%

Sub-Task 2B: Culture CD34+ cells; over-express or knockdown key central and peripheral node genes in SDS networks; assess myeloid/neutrophil phenotypes in myelopoiesis culture ex vivo. (Start date 9/30/2015; duration 24 months
Milestone: Identify SDS myeloid phenotypes ex vivo; define genes responsible for myeloid phenotypes; functionally annotate microRNA-dependent and -independent pathways explaining SDS pathogenesis.

Percent completion: 20%

Sub-Task 2C: Quantify microRNA activity in SDS myeloid progenitor cells; perform microarray Expression profiling of microRNAs in SDS myeloid progenitor cells; re-evaluate microRNA target predictions accounting for microRNA expression changes in affected SDS progenitor cells. (Start date 9/30/2016; duration 12 months)

Milestones: Quantify microRNA activity in SDS myeloid progenitors; discern SDS pathways that are affected by reduced microRNA activity and/or altered microRNA expression

Percent completion: 0%

What was accomplished under these goals?

Patients with SDS suffer from complex and unstable hematopoietic defects. Neutropenia is most common in early stages of the disease, with some patients progressing to pan-anemia, thrombocytopenia or MDS/AML. Due to the complexity of clinical presentation and the rarity and heterogeneity of bone marrow progenitors, the affected cell types and altered genetic networks *in vivo* remain unknown. We applied single cell RNA-seq technology to analyze bone marrow progenitors from SDS patients enrolled in a longitudinal study with our collaborators at Children's Hospital Boston.

Aim 1. We performed single cell RNA-seq on CD34+ hematopoietic stem and progenitor cells (HSPC) from two normal donors (n=95 cells) and four SDS patients (n=176). Data analysis from an additional 200 normal and 200 SDS cells is ongoing. CD34+ cells comprise several stages of hematopoietic development, which we reasoned could be distinguished by transcriptional signatures. To derive these signatures, we performed bulk RNA-seq of FACS-purified hematopoietic stem cells (HSC), multipotent progenitors (MPP), common myeloid progenitors (CMP), granulocyte-monocyte progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) ((1), Fig. 1). We then applied tSNE to cluster HSPC based on these signatures and generate the first single cell maps of early human hematopoiesis (Fig. 2). Expression patterns of hematopoietic transcription factors validate that stratification of single HSPC using our method was consistent with the known hematopoietic developmental trajectory (Fig. 3). The map itself reveals that 1) single HSPCs fall along a continuum of development, illustrating that hematopoiesis is not a series of discrete steps but a continuous process; and 2) SDS and normal cells are similarly distributed along the developmental trajectory, though SDS marrow had a higher proportion of cells at the HSC/MPP stage.

Bioinformatic analyses for the identification of SDS-specific gene expression signatures are ongoing. We are applying single cell statistical frameworks such as MAST, SCDE and DiffCoEx to identify altered patterns of gene expression (2–4). MAST is a differential expression tool that revealed significant upregulation of 34 genes and downregulation of 53 genes in SDS cells ($|\log2(\text{fold change})|\geq1$, $q<0.1$). The upregulated gene set was strongly enriched for the "TNF-alpha signaling via NF-kB" pathway (http://software.broadinstitute.org/gsea/msigdb/, $q=1.34\text{x}10^{-11}$). Nine of the 34 upregulated genes were annotated to this pathway: AREG, FOS, FOSL2, ID2, PDE4B, NAMPT, NR4A1, NR4A2 and NR4A3. We overlaid the summed expression of these genes onto single cell maps from normal donors and SDS patients, and found that they are primarily upregulated in the HSC/MPP cells that are overrepresented in SDS bone marrow (Fig. 4A). Moreover, their expression is positively correlated in SDS cells (Fig. 4B), which may indicate activation of an upstream regulator of NF-kB signaling. Interestingly, high levels of TNF-alpha have been observed in the serum of patients with myelodysplastic syndrome and Fanconi anemia, but our data is the first to suggest a link between inflammation and marrow failure in the context of SDS.

Aim 2. In our initial proposal we planned to validate SDS gene expression changes in primary cultures of normal and SDS CD34+ cells. However, primary hematopoietic cells have limited lifespan and yield little biomass in culture. Thus, we will annotate candidate genes in patient-derived iPSC and then choose a select few to confirm in primary culture. Towards this end, we are collaborating with Dr. Akiko Shimamura, a leading expert in SDS who recently relocated her lab to Boston. Dr. Shimamura has derived iPSC lines from SDS patients and worked out conditions for their hematopoietic differentiation *in vitro*. This work was funded by the Department of Defense, and detailed results have been included in her recent progress report. In addition to the lines derived by Dr. Shimamura, we have also derived iPSC from two of the patients sequenced in Aim 1 that will perfectly recapitulate their genetic background *in vitro*.

Because we are specifically interested in validating the TNF-alpha signature in iPSC-derived HSC, we tested for the presence of CD34+CD90+ HSC relative to CD34+CD90- progenitors over a course of *in vitro* hematopoietic differentiation (Fig. 5). On day 8, CD90+ HSC dominate the CD34+ population, with CD34+CD90- progenitors arising by day 13. Though SDS cultures contain more HSC than normal on day 8, their frequency is reduced by day 13 coinciding with fewer CD34+CD90- progenitors. These data are consistent with Dr. Shimamura's findings that hematopoietic differentiation is impaired in SDS iPSC ((5), and progress report), and further implicate increased apoptosis or impaired self-renewal of HSC as the underlying mechanism. In the coming year we will perform bulk and single cell RNA-sequencing of iPSC-derived HSPC to confirm that they recapitulate gene expression signatures of SDS. Additionally, we will test the ability of TNF-alpha and NF-kB inhibitors to rescue impaired hematopoietic differentiation of SDS iPSC. Finally, we will manipulate expression of selected candidate genes in appropriate HSPC subsets using lineage-restricted promoters and assess consequences on differentiation potential, survival and proliferation of normal and SDS iPSC. These experiments will link gene expression changes during a specific stage of hematopoietic development to bone marrow failure in SDS. Similar work is ongoing in a mouse model of SDS through a collaboration with Dr. David Scadden's lab.

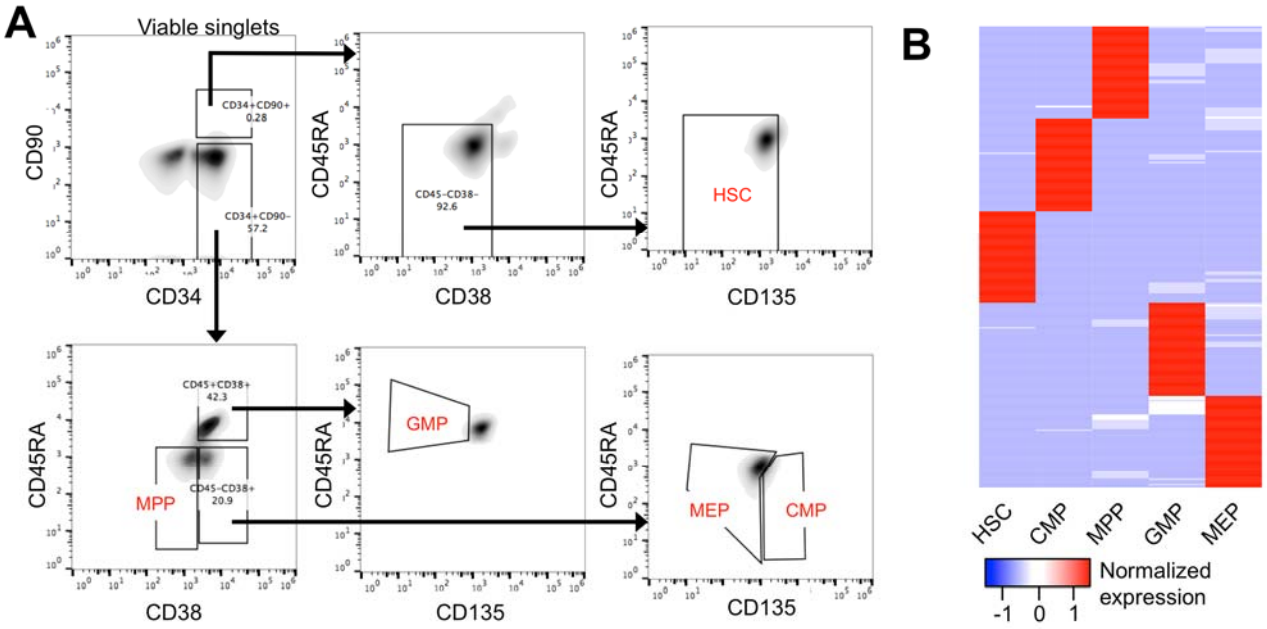

Fig. 1. Transcriptional signatures of early hematopoietic development*. (A) Purification scheme for CD34+ HSPC. (B) Heatmap of 250 genes that are exclusively expressed in HSC, MPP, CMP, GMP and MEP.*

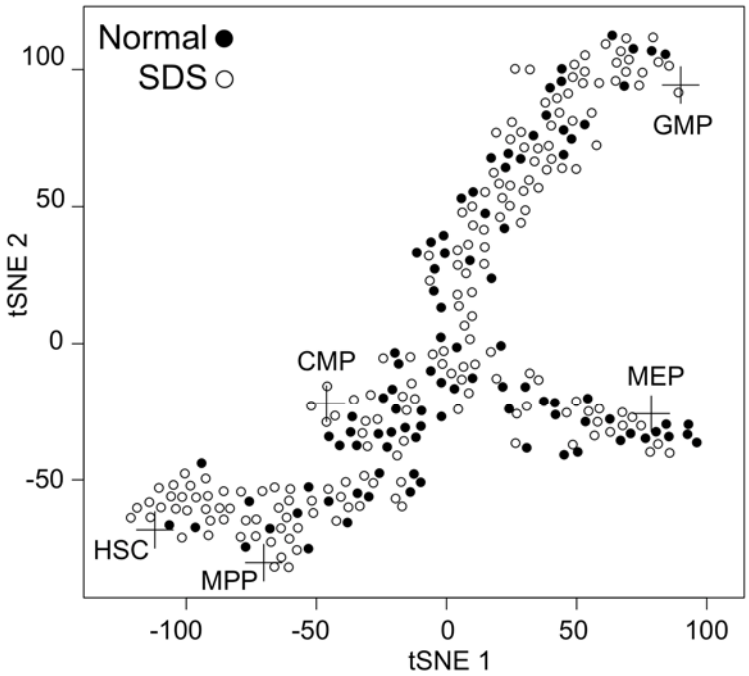

Fig. 2. Transcriptional roadmap of early hematopoietic development at single cell resolution. *Single HSPC from normal and SDS patients were clustered according to the expression of lineage signature genes.*

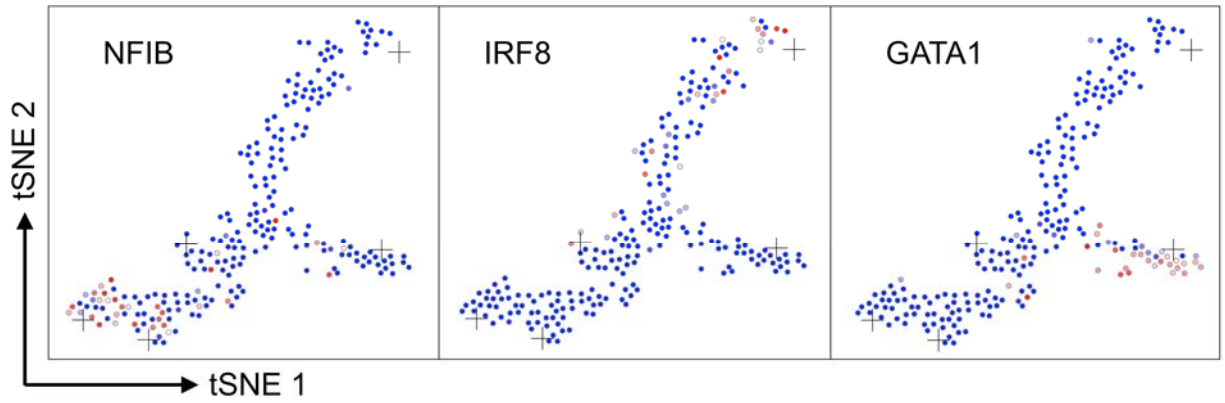

Fig. 3. Lineage-restricted transcription factor expression validates single cell ontogenies. *NFIB, IRF8 and GATA1 are transcription factors enriched in HSC, GMP and MEP, respectively.*

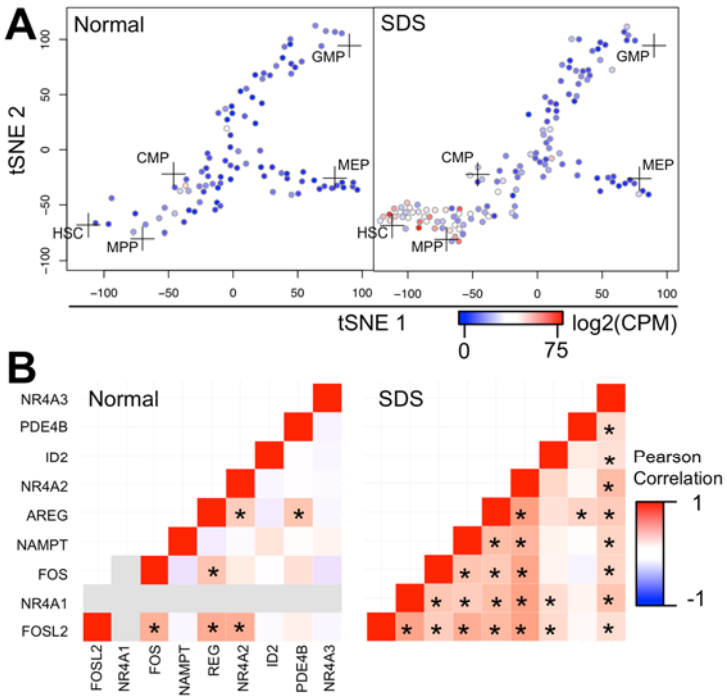

Fig. 4. Activation of TNF-α signaling via NF-κB in SDS HSC/MPP. *(A) Single HSPC from normal or SDS patients were clustered as in Figure 1. The color scale represents summed read counts for upregulated genes in "TNF-α signaling via NF-κB". (B) Positive correlation of the upregulated gene set in SDS cells. NR4A1 was not expressed in any normal cells.*

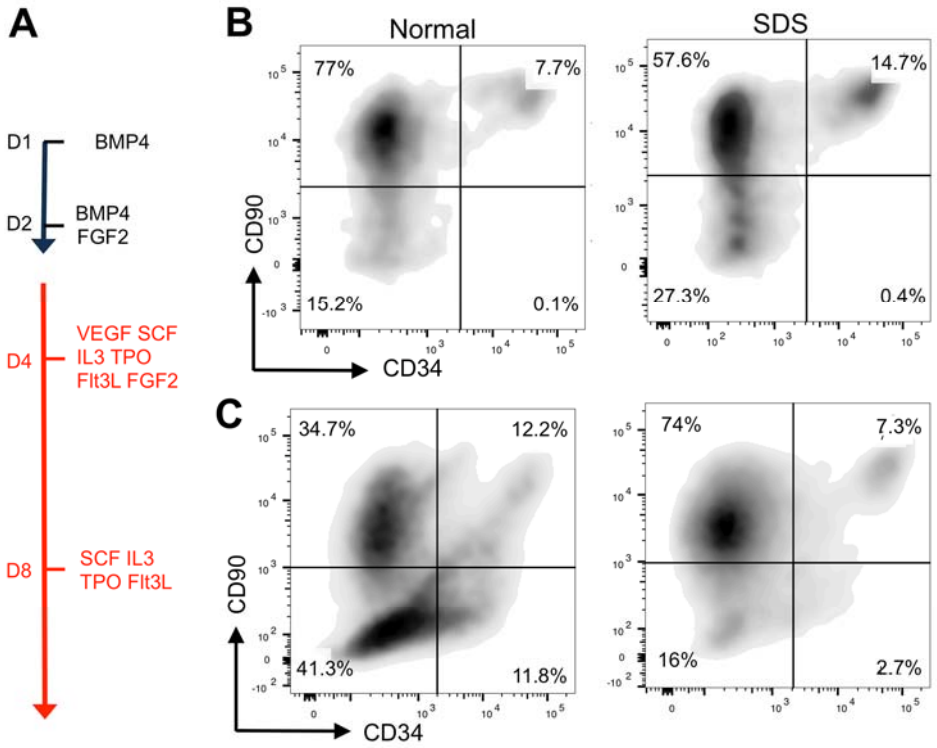

Fig. 5. Reduced frequency of CD34+ HSPC in SDS iPSC cultures. *(A) Timeline of cytokine supplementation to derive hematopoietic progenitors from iPSC. Stage 1 (black arrow, D1-3) promotes embryoid body (EB) formation from iPSC. Stage 2 (red arrow, D4-18) promotes hematopoietic differentiation of EB. iPSC were harvested and analyzed by FACS on (B) D8 and (C) D13.*

REFERENCES

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What opportunities for training and professional development has the project provided?

A postdoctoral fellow in the lab, Dr. Cailin Joyce, attended two meetings in the past reporting period: Single Cell Genomics (Utrecht, Netherlands, September 2015) and the Harvard School of Public Health Program in Quantitative Genetics Annual Symposium (Boston, MA, November 2015). Dr. Joyce has also prepared a K99/R00 application proposing an extension of this work in her independent laboratory. Though the application was not funded, it was met with enthusiasm. She is currently preparing similar applications for the ASH Scholar Award and ASH-EHA Translational Research Training in Hematology Program.

How were the results disseminated to communities of interest?

Dr. Joyce presented posters at both meetings she attended and has given talks locally for the Department of Cancer Immunology and Virology (DFCI), the Department of Microbiology and Immunobiology (HMS) and the Division of Immunology (HMS).

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we will complete Smart-seq and subsequent data analysis for the 6 SDS patients and 6 normal donors proposed in Aim 1. We will also perform Drop-seq on at least two normal and two SDS samples to increase our statistical power for the analysis of rare and transient HSPC subpopulations. We will test whether the iPSC and mouse model systems we have established in collaboration with the Shimamura and Scadden labs, respectively, recapitulate SDS gene expression signatures. We will test candidate genes in the most appropriate model(s) and validate the functional role of high-priority candidates in primary CD34+ cultures. Finally, we will generate microRNA expression profiles from normal and SDS HSPCs that will be mapped onto mRNA expression profiles to test the novel hypothesis that microRNA activity is reduced in SDS.

Impact

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

Changes/Problems

Changes in approach and reasons for change

1) We will implement high-throughput Drop-seq on normal and SDS HSPC in parallel with Smart-seq to enable transcriptional profiling of thousands of cells at reduced cost. These are complementary sequencing approaches that will enable a robust analysis of SDS at single cell resolution.
2) We have acquired a new collaborator to assist us with the derivation and differentiation of human iPSC and the enrollment of new SDS patients in our study. Dr. Akiko Shimamura is a physician-scientist and leading expert in SDS who recently became the director of Bone Marrow Failure and Myelodysplastic Syndrome Programs at DFCI.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
None of the changes described above will result in >25% change in budget allocation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Products

Publications, conference papers, and presentations

Joyce CE, Li S, Hofmann I, Nusbaum C, Sieff C, Mason CE, Novina CD. "Single cell transcriptomic analysis of hematopoietic dysfunction in Shwachman-Diamond Syndrome". Poster, Keystone Hematopoiesis, Keystone, CO, 2015.

Joyce CE, Jiang L, Hofmann I, Nusbaum C, Sieff C, Yuan GC, Novina CD. "Lineage-restricted signatures of bone marrow failure in Shwachman-Diamond Syndrome revealed by single cell RNA sequencing of patient cells". Poster, Program in Quantitative Genetics Symposium, Harvard School of Public Health, 2015.

Journal publications
Nothing to report.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations
Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Carl Novina, M.D. , Ph.D.
Project Role:	Principal Investigator

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.0
Contribution to Project:	Dr. Novina is responsible for the conception and design of all the studies. He supervised their execution, analyzed data, prepared publications related to this work, and presented the findings to the scientific community.
Funding Support:	N/A

Name:	Cailin Joyce, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	7 Calendar Months
Contribution to Project:	Dr. Joyce worked on methods to purify and characterize RNA.
Funding Support:	N/A

Name:	Dolly Thomas, Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6 Calendar Months
Contribution to Project:	Dr. Thomas worked on methods to purify and characterize RNA.
Funding Support:	N/A

Name:	Elaine Yee
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 Calendar Months
Contribution to Project:	Ms. Yee worked on methods to purify and characterize RNA.
Funding Support:	N/A

Name:	Dustin Griesemer
Project Role:	Student

Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6 Calendar Months
Contribution to Project:	Mr. Griesemer worked on methods to purify and characterize RNA.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Organization Name: Boston Children's Hospital
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Broad Institute of Harvard and MIT
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Joslin Diabetes Center
Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Organization Name: Massachusetts General Hospital

Partner's contribution to the project (identify one or more): Please highlight the appropriate ones.

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc.,
- available to project staff);
- Facilities (e.g., project staff use the partner's facilities for project activities);
- Collaboration (e.g., partner's staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and
- Other

Special Reporting Requirements

None